# Preparation of 16S rRNA amplicons for sequencing (Illumina MiSeq - paired end)

# ----- FEM lab, NUIG -----

Here, barcoded, universal bacterial archaeal primers 515F/806R (Caporaso *et al.* 2011), with barcodes and Illumina adaptors, are used to amplify the V4 region of 16S rRNA genes from DNA and/or corresponding transcripts from cDNA. These primer sequences and details of ordering them are available from the Earth Microbiome Project:

<http://www.earthmicrobiome.org/protocols-and-standards/16s/>

High Fidelity taq polymerase should be used to reduce PCR errors and NEB’s High Fidelity Q5 Taq always works really well.

1. Quantify the DNA and/or cDNA of each sample (for cDNA, this is done by quantifying the RNA, normalising to lowest concentratino then reverse transcribing and assuming 1 ng RNA -> 1 ng DNA) using the appropriate Qubit – broad or high sensitivity (Nanodrop not accurate enough).

So, all samples will have the same amount (ng) of template per PCR reaction - > VERY IMPORTANT as not doing this will bias diversity analysis later.

1. Each sample should be amplified in triplicate to reduce PCR based bias (Goodrich *et al.* 2014) with each 25 μl reaction containing:
   1. 1 x Q5 Reaction Buffer (NEB);
   2. 0.4 U Q5 High-Fidelity DNA Polymerase;
   3. 200 µM of dNTPs;
   4. 0.4 µM of each primer and
   5. X µg of genomic DNA or cDNA
2. Negative and positive controls should be systematically included
3. PCR conditions as follows:
   1. 30 seconds hot start at 98 °C followed by
   2. 25 cycles of:
      1. 10 seconds denaturation at 96 °C;
      2. 30 seconds annealing at 50 °C and
      3. 30 seconds elongation at 72 °C, followed by a
   3. final extension of 2 minutes at 72 °C.
4. Pool triplicate amplicons per sample and run a few μl on a 1.5 % agarose gel.
5. If you have non-specific bands: (see note below)

Load the remainder of each sample on a 1.5 or 2 % agarose gel and use a gel purification to excise the correct size amplicons (~300 bp) (Promega SV Gel Wizard works well).

1. Elute purified amplicons in nuclease-free water and quantify using the High Sensitivity dsDNA quantification kit (Qubit).
2. Normalise amplicons from each sample to equimolar concentrations and pool to form your final amplicon library.
3. Quantify (Qubit) and check purity of library on Nanodrop – sometimes gel purification kits leaves traces of salts (guanidine?) which will give low 260/230 ratio. This can mess with sequencing so if this is the case, then re-purify the pooled library on a PCR purification column (eg Qiagen QiaQuick).

**Note: Non specific bands**

If all your samples look like they have the same amount of non-specific bands, it’s possible to gel purify after pooling all equimolar amounts of samples for your library (ie one gel purification). BUT, if some samples look like they have more non-specific bands than others, then samples must be gel purified individually. This is because the Qubit will quantify all the DNA present, regardless of band size. So samples that had more non-specific bands will end up with less than their ‘quota’ of correct sized amplicon if gel excision is performed on the final, pooled library.

**Extra information on primers:**

Between the labs of Dr Abram and Prof O’Flaherty there are 73 barcoded primers (#0 to # 72 of 806R) belonging to the ‘older’ version of primers where the reverse (806R) primers are barcoded and both forward (515F) and reverse have the Illumina adaptors (see Earth Microbiome project).

**Recommended companies for primers:**

MWG Eurofins

Eurogentec

-> both been used and the primers provided worked perfectly for amplicon sequencing.

**Recommended sequencing companies:**

Research and Testing laboratories, Texas

Centre for Genomic Research, University of Liverpool.

Protocol written by C Thorn; 2017